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(54) Title: METHOD OF BINDING A COMPOUND TO A SENSOR SURFACE

(57) Abstract: The present invention relates to a method of non-covalently binding a compound to a sensor surface, said method comprising the step of adsorbing a hydrophobin-like substance to at least a part said sensor surface (which part preferably comprises an electrode). Furthermore, the invention provides sensor provided with a surface at least a part of which is provided with a coating of a hydrophobin-like substance comprising a non-covalently bonded compound.

Title: Method of binding a compound to a sensor surface

The present invention relates to a method of binding a compound to at least a part of a sensor surface (which part preferably comprises an electrode), said method comprising the step of adsorbing a hydrophobin-like substance to said sensor surface.

Classically, hydrophobins are a class of small secreted cysteine-rich proteins of fungi or proteins of bacteria that assemble into amphipathic films when confronted with hydrophilic-hydrophobic interfaces. Some hydrophobins form unstable, others extremely stable, amphipathic films. By assembling at a cell wall-air interface some have been shown to provide for a hydrophobic surface, which has the ultrastructural appearance of rodlets as on aerial hyphae and spores. Some hydrophobins have been shown to assemble into amphipathic films at interfaces between water and oils, or hydrophobic solids, and may be involved in adherence phenomena. It appears that hydrophobins are among the most abundantly produced proteins of fungi, and individual species may contain several genes producing divergent hydrophobins, possibly tailored for specific purposes. Hydrophobins have now been implicated in various developmental processes, such as formation of aerial hyphae, fruit bodies and conidia, and may play essential roles in fungal ecology, including spore dissemination, pathogenesis and symbiosis. Hydrophobins fulfill a broad spectrum of functions in fungal growth and development. For instance, they are involved in formation of hydrophobic aerial structures (e.g. aerial hyphae and fruiting bodies) and mediate attachment of hyphae to hydrophobic surfaces resulting in morphogenetic signals. The mechanisms underlying these functions is based on the property of hydrophobins to self-assemble at hydrophilic-hydrophobic interfaces into amphipathic films. Hydrophobins secreted by submerged hyphae will diffuse in the aqueous environment and may self-assemble at the interface of the medium and the air. This is accompanied by a huge drop in water surface tension, enabling hyphae to breach the interface and to grow into the air. On the other hand, hydrophobins secreted by hyphae that contact a hydrophobic environment will self-assemble at the hyphal surface. The hydrophilic side of the amphipathic film interacts with the hydrophilic polysaccharides of the cell wall, while the hydrophobic side becomes exposed to the hydrophobic environment. Aerial hyphae and spores thus become hydrophobic, while hyphae that grow over a hydrophobic substrate firmly attach to it. Hydrophobins are thus active in the environment of the fungus and at the hyphal surface. Moreover, they also function within the matrix of the cell wall

The term "hydrophobin-like substance" as used herein refers to an essentially isolated or purified amphipathic protein capable of coating a surface, rendering a hydrophobic surface essentially hydrophilic, or, vice versa, a hydrophilic surface essentially hydrophobic, and comprises not only hydrophobins as isolated from nature and substantially free of other fungal substances such as carbohydrate polymers like schyzophylan, but also includes substances that can be obtained by chemically modifying classically known hydrophobins or by genetically modifying hydrophobin genes to obtain genetically modified proteins not at present available from nature, still having the desired amphipathic characteristics. Classically known hydrophobins (see for example WO 96/41882 which also provides guidance to obtain genetically modified hydrophobin-like substances) commonly are proteins with a length of up to 125 amino acids, with a conserved sequence X_n-C-X_{5-9}-C-C-X_{11-39}-C-X_{8-23}-C-X_{5-9}-C-C-X_{6-18}-C-X_m wherein X, of course, represents any amino acid, and n and m, of course, independently represent an integer as disclosed by Wessels et al. (ref. 8). Most classical hydrophobins contain the eight conserved cysteine residues that form four disulphide bridges. However, when the disulphide bridges of a hydrophobin are reduced by chemical modification and the sulfhydryl groups blocked with for example iodoacetamide the protein assembles in water in the absence of a hydrophilic-hydrophobic interface. The structure is indistinguishable from that of native hydrophobin assembled at the water-air interface. Apparently, the disulphide bridges of hydrophobins keep monomers soluble in water e.g. within the cell in which they are produced or in the medium, allowing self-assembly at a hydrophilic-hydrophobic interface but are not necessary to provide for its amphiphatic character per se.

Class I and class II hydrophobins are known, each at about 100 amino acids in length, having characteristic hydropathy patterns. Most, but not all, contain eight conserved cysteine residues that form intramolecular disulphide bridges. Hydrophobins may be glycosylated, but the characteristic amphipathic properties of these proteins can be solely attributed to their amino acid sequences. Although the amino acid sequences of class II hydrophobins are relatively well conserved, those of the class I hydrophobins show a low homology. It would be hard, if not impossible, to design universal primers to pick up class I hydrophobin genes by for example polymerase chain reaction.

Indeed, all hydrophobins that have been physically isolated self-assemble at hydrophilic-hydrophobic interfaces into amphipathic membranes. One side of the hydrophobin membrane is moderately to highly hydrophilic (water contact angles below 90°, for example ranging between 22° and 63°), while the other side exposes a surface

Congo Red. Both dyes can be used as probes to discriminate between the alpha-helical state and the beta-sheet state, each having a high propensity for beta-sheet state but no or lower propensity for alpha-helix state or soluble hydrophobin-like substance. In addition, SC3 and amyloid proteins self-assemble via intermediates and only above a critical concentration. It was suggested that amyloid fibril formation is common to many, if not all, polypeptide chains. However, because formation of amyloid fibrils is accompanied by loss of function or even disease (e.g. Alzheimer's disease), evolution would have selected against the propensity to form such fibrils. Yet, one or two mutation(s) in a protein suffice to considerably increase the tendency to form amyloid fibrils. To our knowledge, hydrophobins are the first example of functional amyloids, with multiple functions in fungal development. Recently, it was found that the four disulfide bridges of the SC3 hydrophobin are essential to prevent the protein from forming the amyloid structures in the absence of a hydrophilic-hydrophobic interface. When the disulphide bridges were reduced and the sulfhydryl groups blocked with iodoacetamide, the protein spontaneously assembled in water. Its structure was then indistinguishable from that of native SC3 assembled at the water-air interface. Apparently, the disulphide bridges of hydrophobins keep monomers soluble in water (e.g. within the cell or in the medium) and thus prevent precocious self-assembly. This would explain why in nature most hydrophobins have eight conserved cysteine residues.

Hydrophobins belong to the most surface-active molecules. With a maximal lowering of the water surface tension from 72 to 24 mJ m^{-2} at 50 µg ml^{-1}, SC3 is the most surface-active protein known. Other hydrophobins are also highly surface active. Their surface-lowering activities are at least similar to those of traditional biosurfactants. In contrast to these surfactants, surface activity is not dependent on a lipid conjugate but is solely caused by the amino acid sequence. Moreover, while the maximal lowering of the surface tension by the traditional surfactants is attained within seconds, it takes minutes to hours in the case of class I hydrophobins. This is explained by the fact that hydrophobins lower the water surface only after self-assembly that is accompanied by conformational changes in the molecule.

Despite the fact that hydrophobins have diverged considerably, their gross properties are similar. This flexibility is also illustrated by the fact that removing 25 out of 31 amino acids preceding the first cysteine residue of the SC3 hydrophobin to generate truncated SC3 by genetic engineering only affected the wettability of the hydrophilic side of the assembled hydrophobin. A most remarkable hydrophobin is the trihydrophobin CFTH1 of *C. fusiformis*. It contains three class II hydrophobin-like

The invention provides a method of providing a sensor surface with a compound, said method comprising the steps of coating at least a part of the surface of the sensor with a hydrophobin-like substance and contacting the compound with the coated hydrophobin-like substance to form a coating comprising said compound in a non-covalently bound form, or at least not covalently bound to a hydrophobin. In one embodiment, as commented upon by Bilewicz et al in J. Phys. Chem. B 2001, 105, 9772-9777 of August 2001 as particularly useful for electrodes, a method according to the present invention is provided in that the compound is chosen from the group consisting of i) an electroactive compound, an ii) a compound capable of being converted into an electroactive compound, said method comprising the steps of coating the electrode with a hydrophobin-like substance, and contacting the compound with the hydrophobin-like substance to form a coating containing said compound in a non-covalently bound form.

A particular type of sensor is the electrochemical sensor, comprising an electrode as the sensor surface. Coating an electrode with a hydrophobin would in general result in reduced access of electroactive compounds to the surface of said electrode. Surprisingly, we have found that it is possible to non-covalently incorporate a compound in said hydrophobin coating. The compound remains in the hydrophobin coating for a substantial time, as evidenced by experiment. Such a compound becomes incorporated when it has a lower molecular weight than the hydrophobin, preferably less than 2000, and more preferably less than 1000 dalton. The electroactive nature of the compound improves the sensitivity of the electrode in comparison with a hydrophobin coating not containing said compound.

Another application of the invention relates to a method of providing a sensor type possessing favorable features for use as a biosensor, which is a device that incorporates a biological recognition element in close proximity or integrated with the signal transducer, to give an essentially reagentless sensing system specific to a target compound. In the method provided in the invention, the biological recognition elements are non-covalently attached to a biosensor surface in a non-covalent manner via a hydrophobin-like coating. This mode of binding leaves the secondary and tertiary structure of such biological compounds virtually intact and thus allows improved biological recognition compared to covalent binding methods which often alter such structural determinants.

In another embodiment, a method according to the present invention is provided wherein a first (non-covalently attached) compound is a proteinaceous substance with a higher molecular weight than a classically known hydrophobin (i.e. >15 kD)

D,E;
N,Q;
R,K;
I,L,M,V;
F,Y,W.

The term "electro-active" is defined as a compound which can undergo changes in the oxidation state, i.e. undergo a redox reaction. An electro-active compound, such as Q10, azobenzene, Q0 or calixerene, will have a lower molecular weight than the hydrophobin, and more in particular it will have a MW of less than 2000 dalton, and more preferably less than 1000 dalton. Compounds capable of being converted into an electroactive compound are known in the art. They may become electroactive after, for example, irradiation with light. This allows the electroactive compound to be made available at a desired time. Some of the advantages of the use of such a compound are

- more accurate measurements (background measurements can be made before the electroactive compound is released)
- reduced consumption of electroactive compound. Generally, the compound capable of being converted into an electroactive compound (an example of which is calixarene) will have a molecular weight as specified above.

In particular, the compound is a hydrophobic compound or a compound containing a hydrophobic anchor. Such compounds are among the compounds most stably maintained in the hydrophobin coating. It is thought that a planar hydrophobic compound or anchor may be beneficial. In the present application the term "anchor" is understood to mean a part of the compound, said part having a side and/or moiety lacking hydrophilic groups. It is also thought that the absence or a reduced number of negative and/or positive charges is advantageous. If charge is present, it is preferably from weakly acidic or basic groups, which can release or accept a hydrogenium ion to eliminate the charge.

Advantageously, a second compound is bound covalently to the hydrophobin, the second compound being an electroactive compound.

According to an alternative embodiment, said second compound is bound non-covalently to the hydrophobin, through a third compound being an intermediate compound having affinity for the second compound, said second compound being an electroactive compound.

Both these methods allow for a (more) selective measurement. With the ("first") compound being present, good sensitivity is achieved even though the second

might for example use a monoclonal antibody to detect an antigen, or a small synthetic DNA molecule to detect DNA. In a biosensor, a biological recognition (sensing) element is in direct spatial contact with a transducer element, to give a reagentless sensing system specific to a target compound (analyte). Transducers are the physical components of the sensor that respond to the products of the biosensing process, which may be optical, electrochemical, thermometric, piezoelectric or magnetic, and outputs the response in a form that can be amplified, stored, or displayed. The biological recognition element may be a biological material or a biomimic (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids etc.). Biological sensing elements have a remarkable ability to distinguish between the analyte of interest and similar substances with great accuracy. Biosensing occurs only when the analyte is recognized specifically by the biological element. It is preferred that the biological elements are bound to the sensor surface in a non-covalent manner, as is the case in the method provided in the invention, leaving secondary and tertiary structures of such biological compounds virtually intact and thus allowing optimal biological recognition.

For a given analyte-recognition element reaction, several transduction schemes may be applicable. Amperometric devices detect changes in current as constant potential. Conductimetric devices detect changes in conductivity between two electrodes. Potentiometric devices detect changes in potential at constant current (usually zero). Optical transducers can be subdivided into two modes (extrinsic and intrinsic) according to the optical configuration. In the intrinsic mode, the incident wave is not directed through the bulk sample, but propagates along a wave guide and interacts with the sample at the surface within the evanescent field. Other surface methods of optical detection of biological recognition are based on modulation of the field excited at the interface between different materials due to incident light. For example, the BIAcore system monitors bio-specific-interactions with a surface plasmon resonance detector to detect minor mass changes at the surface such as antibody binding to a surface-immobilized antigen.

A biosensor is distinguished from a bioanalytical system which requires additional processing steps, such as reagent addition. The reagentless form in most cases is achieved by immobilizing the biological recognition element onto the sensor surface. Advantageously, the biosensor surface is coated with a compound allowing non-covalent attachment of a biological recognition molecule. Where covalent binding often results in loss of reactivity, these compounds bound with a method according to the invention in particular substantially maintain their reactivity towards ligands, anti-

Figure legends

Fig. 1a-c show cyclic voltammograms showing the difference between bare (1) and hydrophobin-modified (2) electrodes (GE, GCE. TMFE respectively);

Fig. 2a,b show cyclic voltammograms using ferrocyanate ions as a probe to check the blocking properties of hydrophobin;

Fig. 3a,b are similar to fig. 1 and show the effect of adsorption of ubiquinone Q10 adsorbed to a GC-electrode (GCE);

Fig. 4 shows the effect of diazobenzene on a cyclic voltammogram for a GCE; and

Fig. 5 is similar to fig. 4, except that the effect of ubiquinone Q0 is shown.

containing 2 μg/ml hydrophobin, and adsorption to the surface was achieved by lifting the electrode up through the interface, or by a horizontal touching of the hydrophobin-covered water surface with the electrode. Figure 1 shows the cyclic voltammograms recorded using the bare (1) and hydrophobin modified (2) electrodes. Figure 1a allows the comparison of the bare (1) and covered (2) GE. The curves are similar in that no decrease of background current is observed, and no peaks appear in the voltammogram. The presence of the hydrophobin layer on the electrode surface is evidenced by the inhibition of the final increase of anodic current due to gold oxidation. HYDPt-1 is inert in a wide range of potentials and does not lead to a decrease of capacity currents which means that the protein layers formed on the electrode are not as dense and highly blocking, as the layers of, *e.g.*, alkanethiols (ref. 2). The extent of blocking is not changed even after 24 hours of self-assembly.

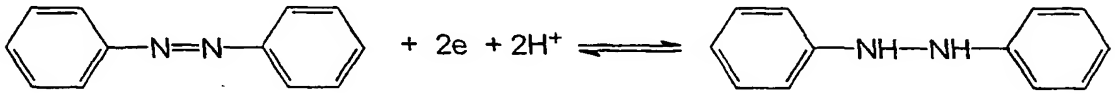
Wessels and Wösten observed that the SC3 hydrophobin had much higher affinity to hydrophobic than to hydrophilic surfaces (ref. 3, 4). Two types of electrodes, GCE and TMFE, were therefore chosen as model hydrophobic surfaces to check the behavior of HYDPt-1. The results of self-assembly are shown in Figures 1b and 1c respectively. In both cases the background currents become much smaller after modification (2), demonstrating that the coverage of GCE and TMFE with HYDPt-1 is much higher, compared to that of the gold substrate. The protein layers are stable and firmly attached to the electrode substrate, as indicated by the GCE voltammogram which does not change over several weeks. In the case of TMFE, high quality films are formed even when the time of self-assembly is decreased from 24 hours to 20 minutes (fig. 1c). The capacity of the modified TMFE is significantly lowered, and the onset of the mercury oxidation current is shifted towards more positive potentials, revealing strong blocking properties of the hydrophobin layer. An additional peak appears in the TMFE voltammogram at -0.58V. This peak corresponds to the reduction of mercury cysteinate formed on the electrode surface upon oxidation of mercury in the presence of cysteine thiol groups present in the protein.

Stability of HYDPt-1 layers in solutions of different pH. The dependence of stability and blocking properties of HYDPt-1 layers on the pH of the solution was checked by recording multiple cyclic voltammograms using all electrodes in solutions of pH 2.2 (citric acid), pH 4.7 (citric acid / LiOH), pH 7.0, pH 10.2 (Tris), and pH 12.1 (LiOH). The HYDPt-1 layer remained well attached to the electrode surfaces in all solutions studied, and the blocking effect on various substrates followed the behavior observed at pH 7.0.

Probing blocking properties of HYDPt-1 layers using ferrocyanate as

trode covered with hydrophobin only. The shape of the curve and the linear dependence of the peak currents on the scan rate points to surface immobilization of ubiquinone. The GCE substrate covered with HYDPt-1 was found to bind Q10 in a very stable way, giving rise to ubiquinone reduction and oxidation signals which remained unchanged for several weeks.

Electroreduction of diazobenzene immobilized on electrodes modified with HYDPt-1. Diazobenzene is a small molecule with a photo- and electroactive azo group, which does not undergo adsorption on a bare glassy carbon electrode. However, when adsorbed on a HYDPt-1 modified electrode, diazobenzene remains stably attached to the surface, even after repeated transfers of the electrode into solutions of different pH and not containing the azocompound. Self-assembly of diazobenzene was carried out from a 1 mM methanol solution. Reduction of diazobenzene can be described as shown in the Scheme 2 :



Scheme 2

Figure 4 shows the cyclic voltammogram of diazobenzene adsorbed for 20 min on the HYDPt-1 - modified electrode, recorded in 0.1 M Tris / $HClO_4$ solution of pH 7.0. Curve 1 was recorded after adsorption of the diazobenzene for the same laps of time, but on the bare GCE. Curve 2 represents the electrode covered with hydrophobin, and curve 3 represents the electrode with both hydrophobin and diazobenzene. The well developed reduction and oxidation peaks do not change upon repeated cycling. The peak currents increase linearly with square root of the scan rate, indicating diffusion control rather than surface - immobilized species. Since the working solution does not contain diazobenzene, this dependence can be understood in terms of diffusion of diazobenzene within the HYDPt-1 layer. Such behavior argues that, in the self-assembly process, the small and hydrophobic diazobenzene molecule penetrates into, and is immobilized in the HYDPt-1 layer.The diazobenzene incorporated into the film is now being studied in our laboratories as a molecular switching device, based on the cis-trans isomerization taking place on UV irradiation. Similar scan rate dependencies were observed for the Q0 molecule, which has the same headgroup as Q10 but does not possess an alkyl chain (Figure 5), and therefore can easily penetrate the HYDPt-1 layer. Curve 1 is a bare electrode in the presence of Q0, and curve 2 an electrode covered with hydrophobin and after adsorption of Q0.

water to remove any unbound hydrophobin; these coatings were referred to as the α-helix state. One hydrophobin coated and one bare electrode of each type was boiled in 2% SDS-solution for 10 min. The SDS-treated electrodes were extensively washed with water. These coatings were referred to as the β-sheet state. The various types of coated and bare electrodes were loaded with electroactive compounds Q10, azobenzene or Q0 as described by Bilewicz et al in J. Phys. Chem. B 2001, 105, 9772-9777 or with a mediator such as methylene blue. The various types of coated and bare electrodes either or not loaded with the electroactive compounds were incubated in LHC of *Cyclotella cryptica*, isolated as indicated in Rhiel et al. (Rhiel E. et al., 1997, Botanica Acta 110, 109-117), at various concentrations for 2 h at 25°C. The electrodes were washed with the appropriate buffers.To assay the activity of the immobilized LHC, the electrodes (in the appropriate buffer) were placed in the dark followed by placing them in daylight and measuring the current. The dark-light cycles were repeated several times on the same day, after 1 day, after 1 week and after 1 month to determine the stability of the immobilized LHC.

Immobilization of CYP2D6 and CYP2C19 on an electrode surface.

A glassy carbon electrode was coated with hydrophobin by emerging the electrode for 15 min in a solution containing hydrophobin (100 µg/ml). The electrode was thereafter thoroughly rinsed. The cytochromes CYP2D6 and CYP2C19 were separately bound on one hydrophobin coated electrode each, by incubation CYP2D6 or CYP2C19 containing solution for 15 minutes and extensively rinsed. The modified electrodes were placed in a medium containing NADPH and the model substrates dextromethorphan and mephenytoin. During the incubation period the potential was measured, which was induced by the contact with the substrates dextromethropan and mephenytoin. The magnitude of the potential reflects the metabolism by the iso-enzymes. After the incubation for 1 hour, the electrodes were removed and the substrates (dextromethorphan and mephenytoin, respectively) and products (dextrorphan and 4-hydroxymephenytoin, respectively) were quantified. The ratio of both was a reflection of the activity of the cytochromes and was correlated to the measured potential.

CLAIMS

1. Method of providing a sensor surface with a compound comprising the steps of coating at least a part of the surface of the sensor with a hydrophobin-like substance and contacting the compound with the coated hydrophobin-like substance to form a non-covalent bond between said hydrophobin-like substance and said compound.

2. Method according to claim 1 wherein said compound is smaller than 15000 dalton.

3. Method according to claim 1 wherein said compound is smaller than 2000 dalton

4. Method according to claim 2 or 3 wherein said compound is incorporated in said coating.

5. Method according to any one of claims 1 to 3 wherein the compound is an electroactive compound.

6. Method according to any one of claims 1 to 3 wherein the compound is capable of being converted into an electroactive compound

7. Method according to claim 1 wherein said compound is larger than 15000 dalton.

8. Method according to claim 1 wherein said compound comprises a proteinaceous substance.

9. Method according to claim 7 or 8 wherein said compound comprises an enzyme.

10. Method according to claim 7 or 8 wherein said compound comprises an antibody.

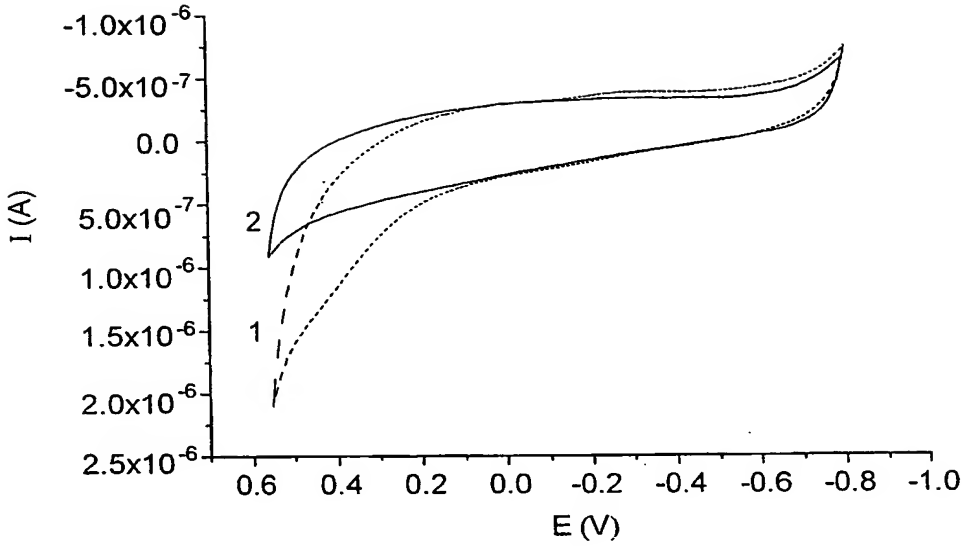
11. Method according to claim 7 or 8 wherein said compound comprises a receptor.

12. Method according to anyone of claims 1 to 11 wherein the compound is at least partly hydrophobic or comprises a hydrophobic anchor.

13. Method according to anyone of claims 1 to 12 wherein a second compound is bound covalently to the hydrophobin-like substance.

14. Method according to anyone of claims 1 to 12 wherein a second compound is bound non-covalently to the hydrophobin-like substance, through a third compound being an intermediate compound having affinity for the second compound.

15. A sensor provided with a surface at least a part of which is provided with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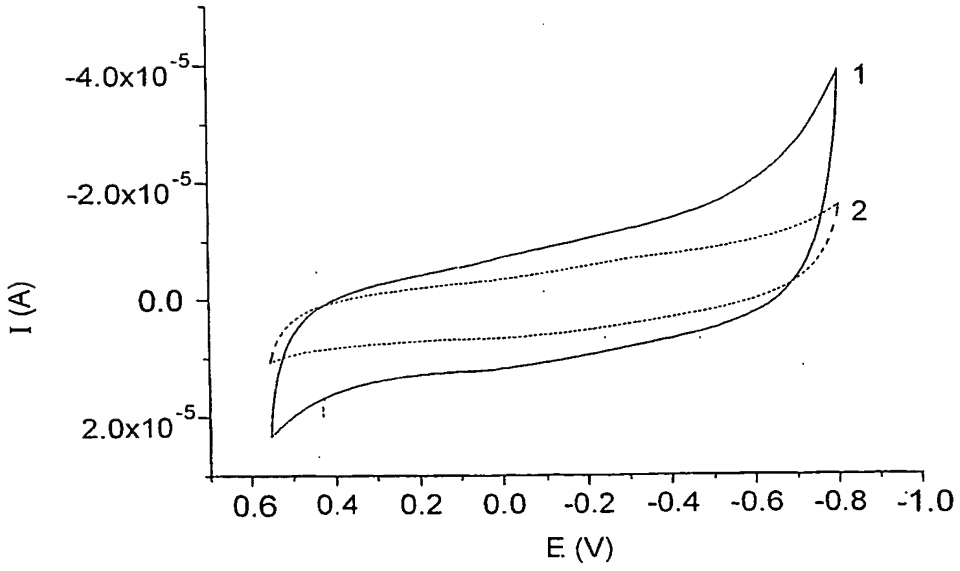
-5.0x10-7
0.0
5.0x10-7
1.0x10-6
1.5x10-6
2.0x10-6
2.5x10-6
I (A)
2
1
0.6 0.4 0.2 0.0 -0.2 -0.4 -0.6 -0.8 -1.0
E (V)

Fig. 1a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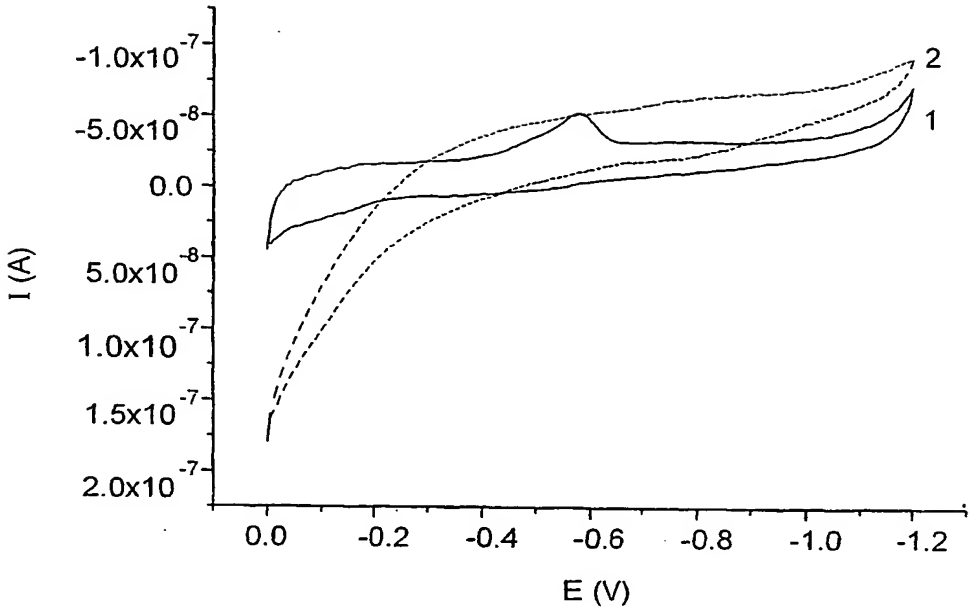
E (V)

Fig. 1b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2.0x10-7
I (A)
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-0.6
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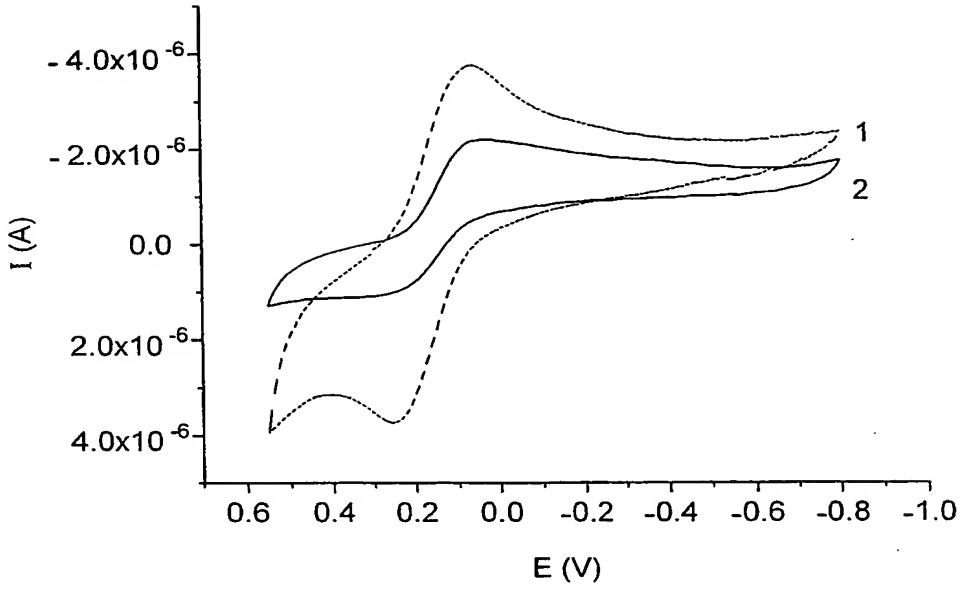
E (V)
2
1

Fig. 1c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E (V)
1
2

Fig. 2a

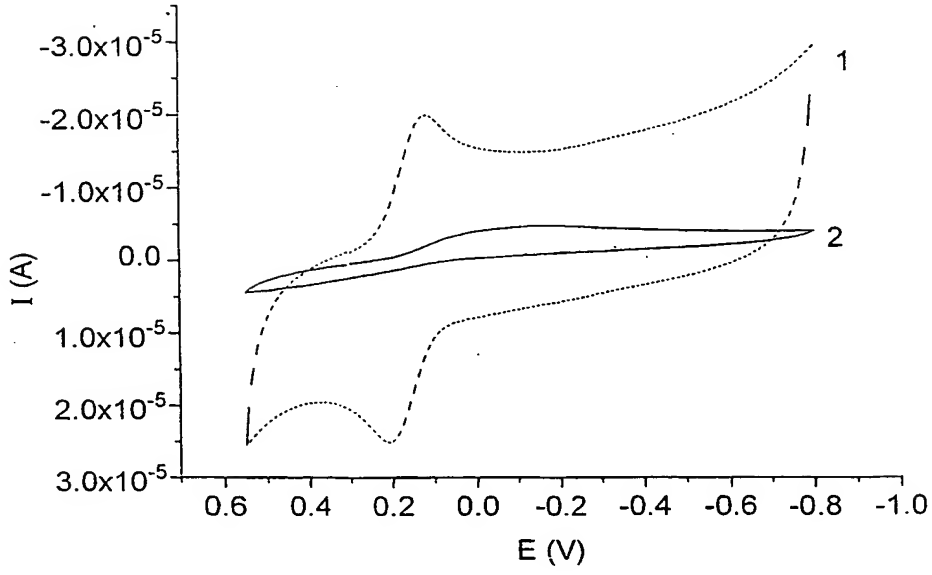
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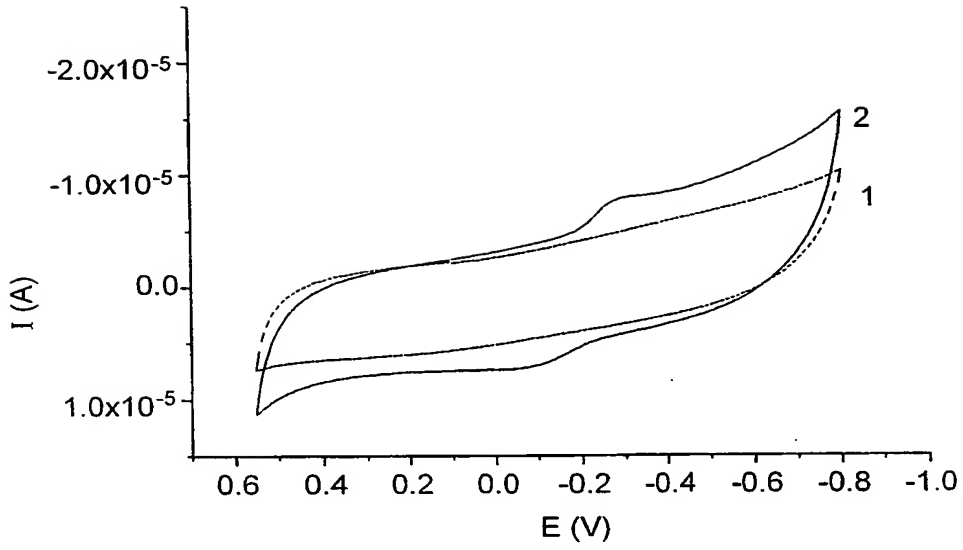
Fig. 2b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E (V)
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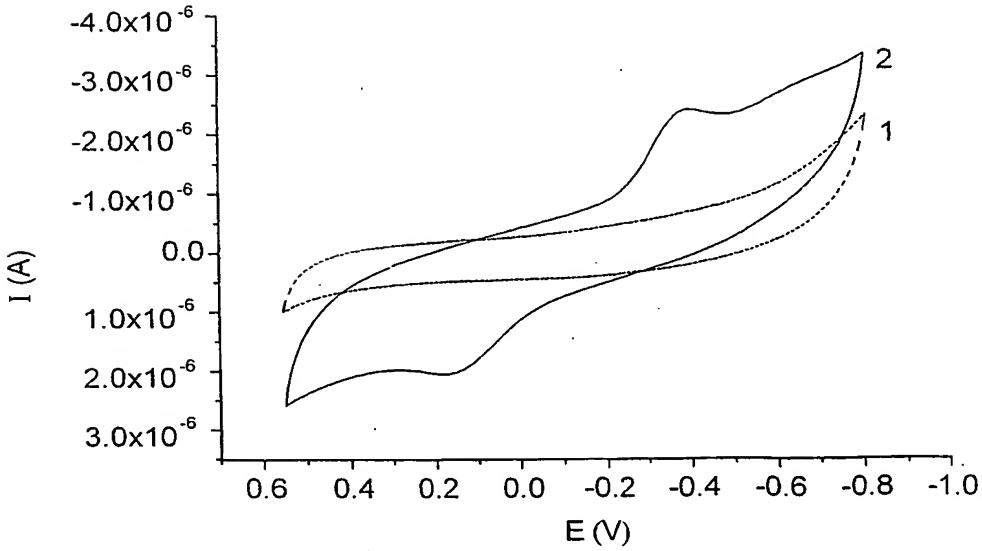
Fig. 3a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I (A)
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E (V)
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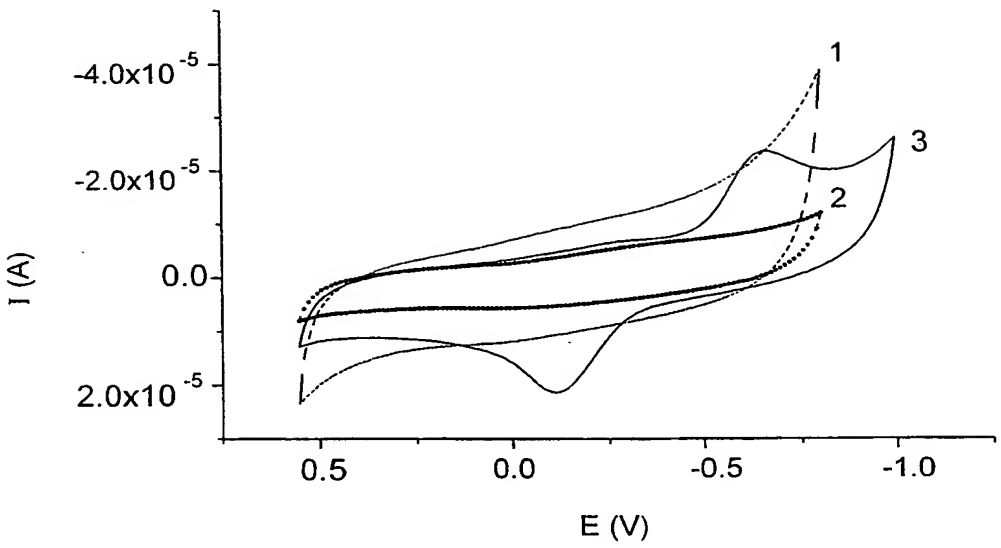
Fig. 3b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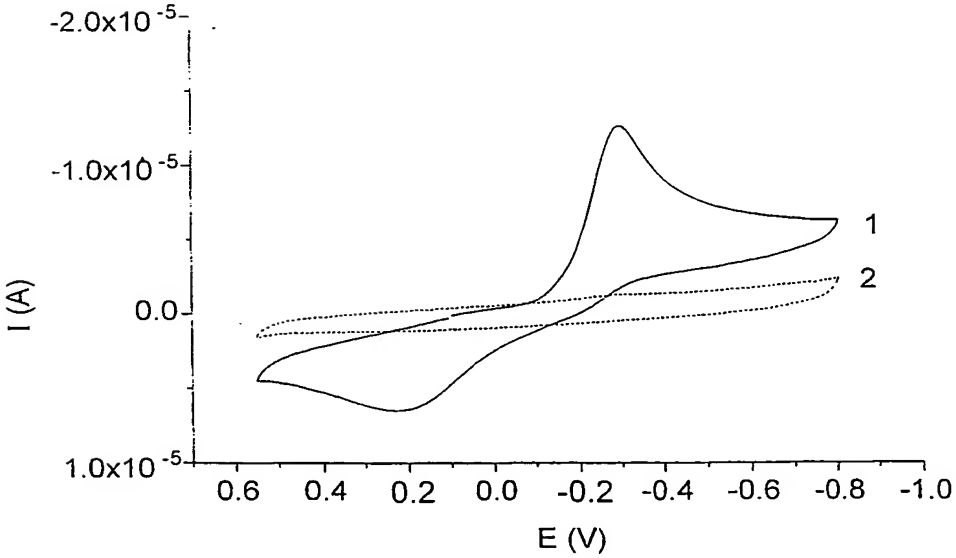
1
2
3

Fig. 4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I (A)
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E (V)
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Fig. 5

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(Source: PCT/NL01/00268)

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